Effect of mineral trioxide aggregate on proliferation of cultured human dental pulp cells

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Abstract

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Aim To investigate the effect of mineral trioxide aggregate (MTA) on the proliferation of human dental pulp (HDP) cells *ex-vivo*.

Methodology Human dental pulp cells were cultured with MTA or calcium hydroxide-containing cement (Dycal) using culture plate inserts. Control cells were cultured with culture plate inserts only. Cell proliferation was measured for up to 14 days using a Cell Counting kit, and the concentration of calcium ions released from the tested materials was assessed using a Calcium E-test kit. To confirm that the effect of MTA was attributable to released calcium ions, cell proliferation was measured in the presence of exogenous calcium chloride as a source of calcium ions while in the absence of MTA.

Results Mineral trioxide aggregate significantly stimulated cell proliferation after 12 days, whereas Dycal had no such effect. The number of calcium ions released from MTA was significantly higher than that released from Dycal. Following the addition of calcium chloride, cell proliferation increased in a dose-dependent manner after 12 days. Moreover, cell proliferation showed a similar pattern whether a given concentration of calcium ions was produced by calcium chloride or by release from MTA.

Conclusions In this *ex-vivo* study, the elution components such as calcium ions from MTA had higher proliferation ability of HDP cells than control and Dycal.

Keywords: calcium ion, cell proliferation, human dental pulp cells, mineral trioxide aggregate, pulp capping.

Introduction

Direct pulp capping is one of the most important endodontic modalities for maintaining dental pulp vitality. It is defined as the treatment of exposed vital pulp by sealing the pulpal wound with a dental material to facilitate the formation of reparative dentine. A final goal of the application of capping materials is to induce the dentinogenic potential of pulp cells (Schröder 1985, Mjör *et al.* 1991, Abedi & Ingle 1995). The dentinogenic potential can result directly from the

biological effects of these materials on pulp cells or indirectly as a part of the wound healing process in traumatized pulp. The healing process of pulp tissue includes odontoblast activation and dentinal bridge formation at the exposed site of dental pulp tissue (Schröder 1985), and undifferentiated cells may replace dead or necrotic odontoblasts (Smith *et al.* 1995).

A number of materials have been used for direct pulp capping, and calcium hydroxide is widely used as a pulp capping agent (Camp *et al.* 2002). The antibacterial effect of this material on infected tissues is of major importance, and experimental observations have shown that reparative dentine formation is induced after pulp capping (Schröder 1972, Cvek 1978). However, clinical observations of direct pulp capping with calcium hydroxide vary (Cvek 1978, Mejàre & Cvek 1993, Barthel *et al.* 2000). Some researchers

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question the use of hard-setting calcium hydroxide in long-term applications because of its degradation over time, tunnel defects through dentinal bridges, and poor sealing ability (Schuurs *et al.* 2000).

Mineral trioxide aggregate (MTA) has excellent sealing ability when used as a sealing material on accidental perforations or as a root-end filling material (Lee *et al.* 1993, Daoudi & Saunders 2002, Weldon *et al.* 2002, Mah *et al.* 2003, Mangin *et al.* 2003). Many *in-vivo* and *ex-vivo* studies have shown that MTA has excellent biocompatibility (Torabinejad *et al.* 1995, Saidon *et al.* 2003). Additionally, when MTA was used for direct pulp capping, it showed better interaction with dental pulp tissue than did calcium hydroxide or acid-etched dentine bonding (Dominguez *et al.* 2003). Newly formed hard tissues were observed in direct contact with MTA in cases of pulp capping *in-vivo* (Ford *et al.* 1996, Andelin *et al.* 2003). Moreover, MTA has been shown to induce less pulp inflammation and more dentine bridge formation compared with calcium hydroxide cement (Faraco & Holland 2001).

However, the effect of MTA on dental pulp tissues is not fully understood at present. In addition, no assessment of the effect of MTA on human dental pulp (HDP) cells has yet been reported. The aim of this study was to evaluate the effect of MTA on the proliferation of HDP cells *ex-vivo*.

Materials and methods

Tissue culture

Four HDP specimens were obtained during tooth extraction from periodontally healthy and noncarious third molars of patients aged 20–27 years. Informed consent was acquired in accordance with the Helsinki Declaration of 1975 and 1983, and the study was also approved by the Ethics Committee of Nihon University School of Dentistry. The teeth were split open, and the pulp tissues were removed under sterile conditions, minced into cubes of 1–2 mm^3 with a surgical knife, and put in 6-well cell culture plates containing α-minimum essential medium (α-MEM; Gibco Invitrogen, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS, Hyclone, South Logan, UT, USA) and antibiotics (50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 100 μg mL^{-1} neomycin; Gibco). The cells were maintained at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. Cells were grown from tissue fragments, collected by trypsinization (0.2% trypsin and 0.02% EDTA;

Gibco), and subcultured. The medium was changed every 2 days, and cell morphology was observed using phase-contrast microscopy to confirm the maintenance of culture condition for HDP cells. The cells were used between the fourth and eighth passages as previously described by Lu *et al.* (2002) and Gruber *et al.* (2004).

Preparation of test materials

Test materials used in this study were MTA (ProRoot, Dentsply Tulsa Dental, Johnson City, TN, USA) and calcium hydroxide-containing cement (Dycal®; Dentsply Caulk, Milford, DE, USA). These materials were prepared according to manufacturers' instructions. Each pellet (diameter, 2 mm; thickness, 0.3 mm) was allowed to set for 24 h at 37 °C in 100% humidity, and placed in α-MEM (0.7 mL) for 3 days with a daily change of medium as previously described by Haglund *et al.* (2003) and Saidon *et al.* (2003).

Cell proliferation in the presence of test materials

The measurement of cell proliferation in the presence of the test materials was performed using 24-well cell culture plates, each well had a culture plate insert with a porous bottom (3 μm pore size) (BD Falcon, Franklin Lakes, NJ, USA). HDP cells were seeded onto the plates at an initial density of approximately 2×10^4 cells per well in 0.7 mL α-MEM containing 10% FBS. The cells were incubated for 24 h to allow adhesion, and then a culture plate insert with one pellet of test material was placed into each well, as shown in Fig. 1. Cells cultured without test material served as controls. The number of cells was determined using a Cell Counting kit (Wako Fine Chemicals, Osaka, Japan) at days 3, 5, 7, 9, 12 and 14. At the time points indicated, the medium was replaced with fresh medium containing 10% (v/v) Cell Counting reagent, and the incubation was continued for 2 h. After incubation, the absorbance of the reaction products was measured at 450 nm with a microtitre plate reader (Titertec Multiskan Plus, Flow Laboratory, McLean, VA, USA). The cell number was calculated from the absorbance value relative to a standard curve.

Measurement of calcium ion release

For evaluation of calcium ion release, test materials were incubated in medium without cells by placing cell culture inserts, each with one pellet of test material, into cell culture wells containing 0.7 mL α-MEM without FBS. The medium was collected at the time
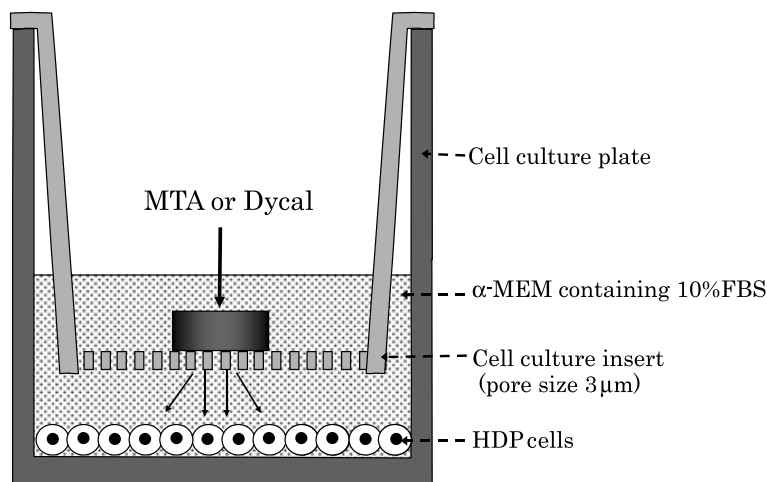
Figure 1 Culture plate with cell culture insert.

points indicated above, and the concentration of calcium ions in the medium was detected using a Calcium E-Test kit (Wako Fine Chemicals). At each time point, 1 mL Calcium E-Test reagent and 2 mL buffer were added to 50 μL of the collected medium, and the absorbance of the reaction products was measured at 610 nm with a microtitre plate reader (Titertec Multiskan Plus). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve.

Effect of exogenous calcium ions on cell proliferation

Human dental pulp cells were cultured in α-MEM containing calcium chloride ($CaCl_2$; Wako Fine Chemicals) as a source of calcium ions at 0–3 mmol L^{-1}. At the time points indicated, the number of cells was determined with a Cell Counting kit as described above.

Statistical analysis

All experiments were performed in triplicate. Each value represents the mean ± SD. Statistical significance was determined using Student's *t*-test when compared with control. Differences with *P*-values <0.05 were considered significant.

Results

Cell proliferation in the presence of test materials

Human dental pulp cells were exposed to MTA or Dycal for up to 14 days. MTA significantly ($P < 0.01$, $P < 0.05$) increased cell proliferation compared with control levels after 12 days in all specimens and after

9 days in specimens 1 and 4. In contrast, Dycal did not affect cell proliferation in any specimen compared with control levels (Fig. 2).

Measurement of calcium ion release

The concentration of calcium ions in the MTA wells was significantly ($P < 0.01$) higher than that in the Dycal wells at all time points (Fig. 3). The concentration was almost constant throughout the experimental period in both the MTA and Dycal wells. The average calcium ion concentrations were 2.1 mmol L^{-1} in the MTA wells and 1.7 mmol L^{-1} in the Dycal wells. The α-MEM medium used in this study contained 1.8 mmol L^{-1} calcium ions. Thus, the calcium ion concentration increased approximately 0.3 mmol L^{-1} in the MTA wells and essentially did not change in the Dycal wells.

Effect of exogenous calcium ions on cell proliferation

Human dental pulp cells were cultured with various concentrations of calcium ions for up to 14 days. When cells were cultured in 0.3 and 3 mmol L^{-1} calcium ions, proliferation was significantly increased in a dose-dependent manner compared with control cell proliferation (Fig. 4). The addition of 0.03 mmol L^{-1} calcium ions increased cell proliferation in a nonsignificant manner. Interestingly, the increase of cell proliferation following the addition of 0.3 mmol L^{-1} calcium ions was similar to that in the presence of MTA.

Discussion

This study was designed to evaluate the effect of MTA on the proliferation of HDP cells using cell culture

Specimen 1

Specimen 3

Specimen 2

Specimen 4

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 *  :  P < 0.05
**  :  P < 0.01
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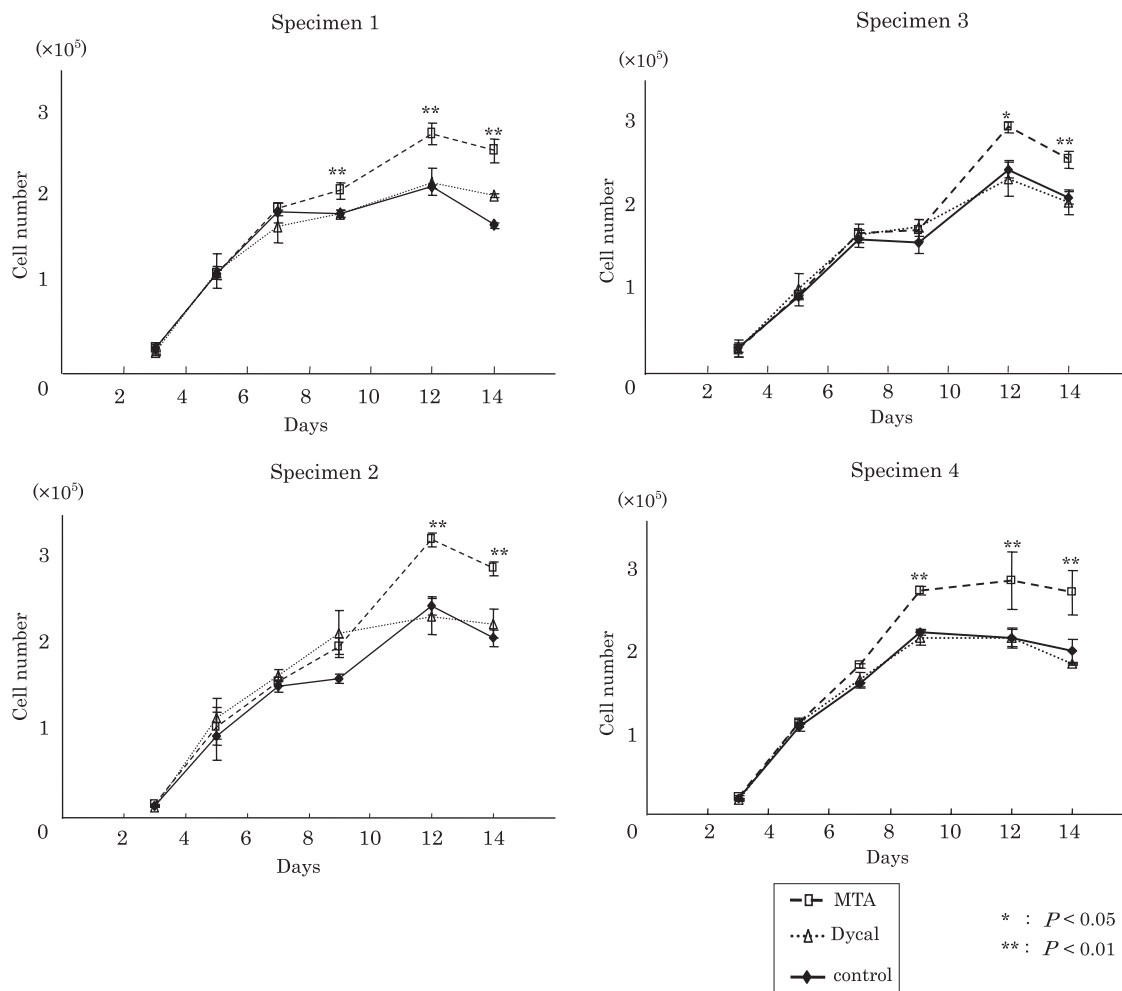
Figure 2 The proliferation of human dental pulp (HDP) cells in the presence of the test materials. The data shown are mean ± SD for three separate experiments. Each specimen was derived from different patients. The asterisk (*P < 0.05; **P < 0.01) indicates statistical significance as compared with controls.

inserts to prevent direct physical interactions between materials and cells. In some *ex-vivo* studies, the test material was placed in direct contact with the cells (Koh *et al.* 1998, Pérez *et al.* 2003, Thomson *et al.* 2003). Pretreated plastic is usually used for optimal cell attachment, and any interruption of the coated surface may inhibit cell attachment and subsequently cell numbers and proliferation, because cells require optimal cell-to-surface and cell-to-cell contact for growth (Koulaouzidou *et al.* 2004). Furthermore, the effect of components, such as calcium ions, that diffuse from the materials was of interest in this study. The insert system is appropriate for evaluating such effects because the material is placed close to the cells without

interfering with the methods used to assess cell proliferation. However, the present *ex-vivo* system has a limitation that the use of an interposed membrane between the materials and the HDP cells allows only for the assessment of the effect of diffusible components of MTA. The size of pellet and the indirect contact between the MTA and the cells also do not reflect clinical usage directly.

Haglund *et al.* (2003) reported that MTA caused the denaturation of adjacent cell and culture medium proteins because of its high pH value during its fresh and setting states. MTA showed favorable biocompatibility with no effect on cell morphology and limited impact on cell proliferation after setting. In this study,
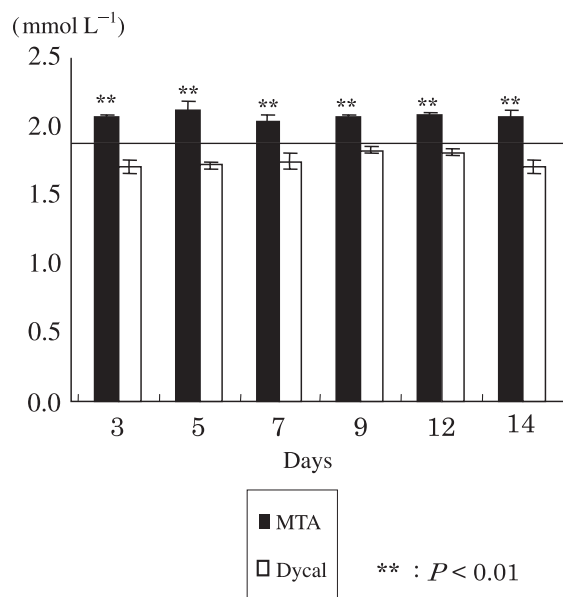
Figure 3 The amount of calcium ions released from the test materials into the culture medium. The data shown are mean ± SD for three separate experiments. The asterisk (**$P < 0.01$) indicates statistical significance as compared with Dycal. Full line shows calcium ions containing in α-MEM (1.8 mmol L^{-1}).

direct contact between the cultured cells and the freshly mixed MTA over a long period (14 days) would have been less meaningful, as cultured cells lack the healing mechanisms of living tissues. Moreover, the chemical properties of freshly mixed MTA could overshadow its important effects after setting. Therefore, materials were immersed in α-MEM for 3 days before using them to avoid the influence of freshly mixed materials in the present *ex-vivo* condition.
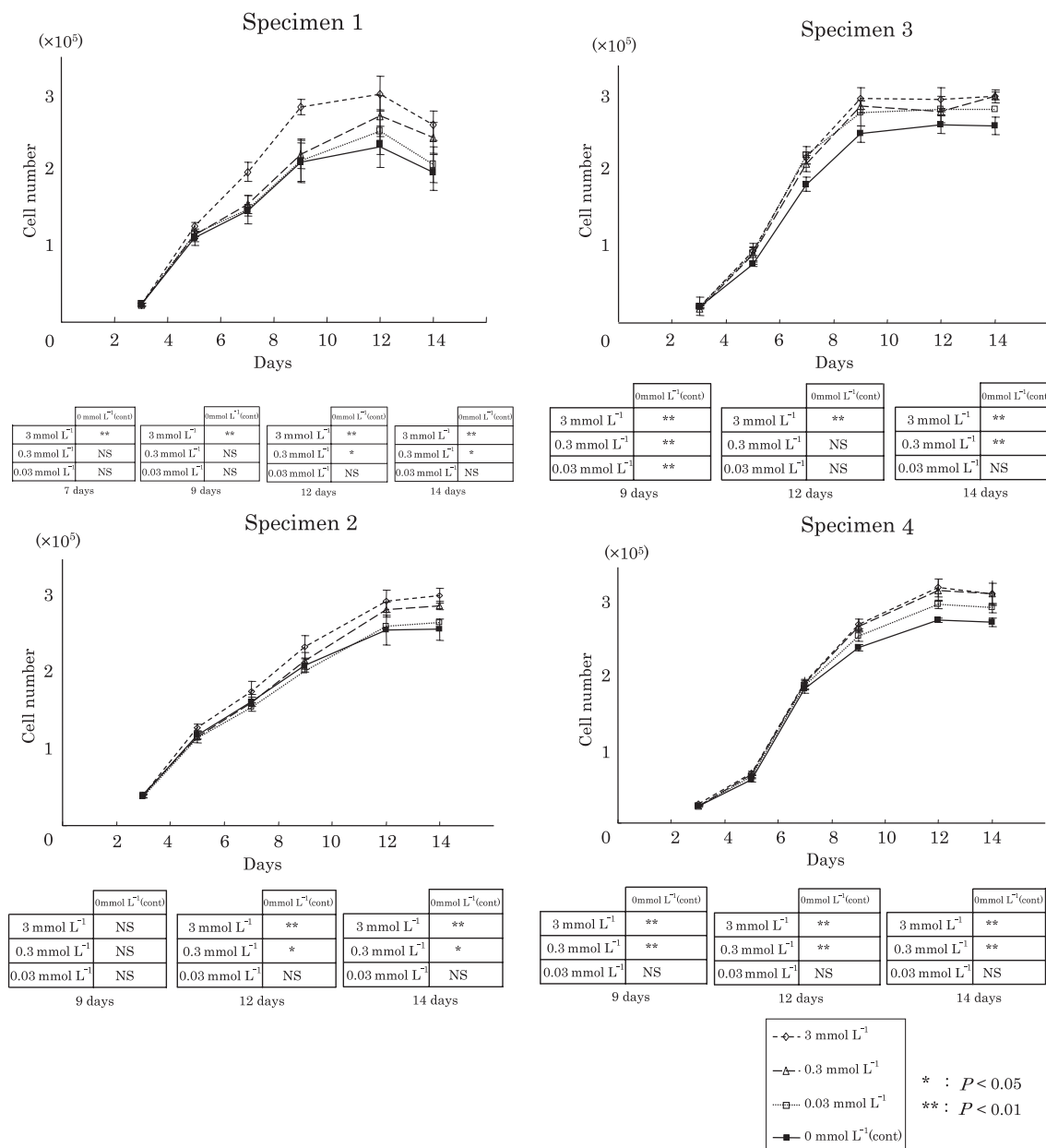
In previous studies, MTA did not induce cell apoptosis, but rather induced a slight increase in the proliferation of mouse odontoblast-like cells (MDPC-23) and undifferentiated pulp cells (OD-21) (Moghaddame-Jafari *et al.* 2005). Koulaouzidou *et al.* (2004) showed that MTA has almost no cytotoxicity to rat pulp cells (RPC-C2A) in a sulforhodamine-B assay. However, the interactions between MTA and pulp cells are not well known, and no evaluation of the effects of MTA on HDP cells has yet been reported. In the present study, MTA enhanced cell proliferation of HDP cells compared with control levels, but Dycal did not affect cell proliferation (Fig. 2). It is difficult to compare directly the present results with those of previous studies owing to different experimental conditions, such

as cell type or culture conditions. However, in agreement with previous studies, the superior biocompatibility of MTA with HDP cells was confirmed.

Fridland & Rosado (2003) reported that calcium was the main component of MTA, based on a chemical analysis of the salts dissolved from MTA in water. Duarte *et al.* (2003) reported that the values for calcium ion release from freshly mixed MTA were higher during the first 3 h and tended to subsequently decrease. Thus, we hypothesized that the release of calcium ions from MTA would induce the proliferation of HDP cells.

As expected, significantly more calcium ions were released from MTA than from Dycal. The α-MEM used in the present study originally contained 1.8 mmol L^{-1} calcium ions, therefore, calcium ions were continuously released from MTA for up to 14 days, even when MTA had been immersed in α-MEM for 3 days before use. On the other hand, the calcium ion concentration in each Dycal well was approximately 1.7 mmol L^{-1}, nearly the same as the concentration in α-MEM medium, throughout the experimental period, indicating that calcium ions were not often released from Dycal in this experiment. Tagger *et al.* (1988) suggested that Dycal released calcium ions for up to 75 min and that no release could be detected after that time. Others have reported some calcium ion release from Dycal (Shubich *et al.* 1978, Tamburić *et al.* 1993). It is difficult to compare directly these findings with the present study because the experimental protocols were different. The failure to detect calcium ion release from Dycal may have resulted from storing Dycal for 24 h after mixing and placing it in α-MEM medium for 3 days before use.

The enhancement of cell proliferation by MTA and continuous release of calcium ions from MTA were confirmed. Therefore, exogenous calcium chloride as a source of calcium ions was added to medium in the absence of MTA on the basis of the hypothesis that the effect of MTA on the increase of cell proliferation is related to the release of calcium ions from MTA. As a result, the calcium chloride increased the proliferation of HDP cells compared with control levels. The amount of calcium ions released from MTA was almost constant in each culture period, and the calcium ion concentration was approximately 0.3 mmol L^{-1}, which is nearly the same as the concentration with the addition of 0.3 mmol L^{-1} calcium chloride. They showed a similar pattern of cell proliferation. Thus, one of the main reasons for MTA-induced proliferation of HDP cells may be the continuous release of calcium ions from MTA.

Specimen 1

	0 mmol L⁻¹(cont)		0mmol L⁻¹(cont)		0mmol L⁻¹(cont)		0mmol L⁻¹(cont)
3 mmol L⁻¹	**	3 mmol L⁻¹	**	3 mmol L⁻¹	**	3 mmol L⁻¹	**
0.3 mmol L⁻¹	NS	0.3 mmol L⁻¹	NS	0.3 mmol L⁻¹	*	0.3 mmol L⁻¹	*
0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS
7 days		9 days		12 days		14 days	

Specimen 3

	0mmol L⁻¹(cont)		0mmol L⁻¹(cont)		0mmol L⁻¹(cont)
3 mmol L⁻¹	**	3 mmol L⁻¹	**	3 mmol L⁻¹	**
0.3 mmol L⁻¹	**	0.3 mmol L⁻¹	NS	0.3 mmol L⁻¹	**
0.03 mmol L⁻¹	**	0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS
9 days		12 days		14 days	

Specimen 2

	0mmol L⁻¹(cont)		0mmol L⁻¹(cont)		0mmol L⁻¹(cont)
3 mmol L⁻¹	NS	3 mmol L⁻¹	**	3 mmol L⁻¹	**
0.3 mmol L⁻¹	NS	0.3 mmol L⁻¹	*	0.3 mmol L⁻¹	*
0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS
9 days		12 days		14 days	

Specimen 4

	0mmol L⁻¹(cont)		0mmol L⁻¹(cont)		0mmol L⁻¹(cont)
3 mmol L⁻¹	**	3 mmol L⁻¹	**	3 mmol L⁻¹	**
0.3 mmol L⁻¹	**	0.3 mmol L⁻¹	**	0.3 mmol L⁻¹	**
0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS	0.03 mmol L⁻¹	NS
9 days		12 days		14 days	

- ◇ - 3 mmol L⁻¹
- △ - 0.3 mmol L⁻¹
- □ - 0.03 mmol L⁻¹
- ■ - 0 mmol L⁻¹(cont)

* : $P < 0.05$
** : $P < 0.01$

Figure 4 The proliferation of human dental pulp (HDP) cells in the presence of various concentrations of exogenous calcium chloride. The data shown are mean ± SD for three separate experiments. Each specimen was derived from different patients. The asterisk (*$P < 0.05$; **$P < 0.01$) indicates statistical significance as compared with controls.

A high ratio of water to powder might be beneficial for the release of calcium hydroxide, however, the amount of water that could be incorporated into the mix was limited by a loss of consistency in the presence of excessive liquid (Fridland & Rosado 2003). Additionally, the physical and chemical properties of MTA may depend on the water-to-powder ratio. As a result,

although a higher proliferation of HDP cells was observed at high concentrations of calcium ions, the mixing of MTA according to the manufacturer's instructions would be ideal.

In this *ex-vivo* study, MTA enhanced the proliferation of HDP cells potentially through the continuous and constant release of calcium ions. MTA is rich in

calcium oxide, which is converted to calcium hydroxide on contact with tissue fluid. The calcium hydroxide separates into calcium and hydroxide ions, resulting in increased pH and calcium ion release. Calcium ions may cross the cell membrane by depolarization or activation of membrane-bound calcium channels, therefore, it is likely that this ion would play a greater role in the reparative process than would the hydroxyl ion (Hunter *et al.* 1998). Calcium ions are necessary for the differentiation and mineralization of pulp cells (Schröder 1985). Torneck *et al.* (1983) pointed out that the presence of large quantities of calcium ions *in-vivo* could activate ATP, which plays a significant role in the mineralization process. Rashid *et al.* (2003) showed that calcium ions specifically modulated osteopontin and bone morphogenetic protein-2 levels during pulp calcification. On the basis of these findings and the present results, the increased proliferation by MTA might be, at least in part, a help to mineralization of HDP cells.

Acknowledgements

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